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cis-Effect of DnaJ on DnaK in ternary complexes with chimeric DnaK/DnaJ-binding peptides

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Abstract Chimeric peptides, comprising a DnaK-binding sequence of L-amino acid residues (motif *k*) and an exclusive DnaJ-binding sequence of D-amino acid residues (motif *j*) connected through a 22-residue linker, were examined as minisubstrates for the DnaK chaperone system. The DnaJ-stimulated ATPase activity of DnaK was three times higher in the presence of the chimeric peptides p*jk* or p*kj* than in the simultaneous presence of the corresponding single-motif peptides ala-p5 (*k* motif) plus D-p5 (*j* motif). Apparently, p*jk* and p*kj* mimic unfolded proteins by forming ternary (ATP·DnaK)·peptide·DnaJ complexes which favor *cis*-interaction of DnaJ with DnaK. Consistent with this interpretation, the specific stimulatory effect of the chimeric peptides was abolished by either single-motif peptide in excess.

Key words: Molecular chaperone; Hsp70; Hsp40; DnaK; DnaJ; Chimeric DnaK/DnaJ-binding peptides

1. Introduction

Hsp70 (70-kDa heat shock protein) molecular chaperones assist a variety of cellular processes, including folding of nascent polypeptide chains, rescue of misfolded proteins, translocation of polypeptide chains through membranes, assembly and disassembly of protein complexes and the control of the biological activity of folded regulatory proteins. The Hsp70 chaperone system of *Escherichia coli* includes DnaK and its two cohort proteins DnaJ, which is an Hsp40 (40-kDa heat shock protein) homolog, and GrpE. DnaK consists of an ATPase domain and a substrate-binding domain [1,2]. The chaperone action of DnaK is driven by the hydrolysis of ATP. DnaJ triggers the hydrolysis of DnaK-bound ATP and thus converts DnaK from the ATP-liganded low-affinity T state to its ADP-liganded high-affinity R state [3]. GrpE accelerates the nucleotide exchange in DnaK and converts DnaK back to its ATP-liganded state [4,5].

Besides stimulating the ATPase activity of DnaK through its J-domain, DnaJ also associates with unfolded polypeptide chains and prevents their aggregation [6–8]. Thus, DnaK and DnaJ may bind to one and the same polypeptide chain to form a ternary complex. The formation of a ternary complex may result in *cis*-interaction of the J-domain of DnaJ with the ATPase domain of DnaK [9,10]. Such *cis*-interaction not only enhances the stimulatory effect of DnaJ on the ATPase activity of DnaK but, as shown recently, is also essential for the chaperone action of the DnaK/DnaJ/GrpE system in refolding unfolded luciferase in vitro [11]. An unfolded polypeptide may enter the chaperone cycle by associating first either with ATP-liganded DnaK or with DnaJ [12]. DnaK interacts with both the backbone and side chains of a peptide substrate [2]; it thus shows binding polarity and admits only L-peptide segments. In contrast, DnaJ has been shown to bind both L- and D-peptides and is assumed to interact only with the side chains of the substrate [13,14]. The differential substrate-binding properties of DnaJ and DnaK make it feasible to design chimeric minisubstrates that mimic unfolded polypeptide chains by favoring the formation of ternary (ATP·DnaK)·substrate ·DnaJ complexes. In this study, we investigated the interaction of DnaK and DnaJ with two chimeric peptides p*kj* and p*jk*, which contain the same DnaK-binding motif (*k*, with L-amino acid residues) and the same exclusive DnaJ-binding motif (*j*, with D-amino acid residues) in different order.

2. Materials and methods

2.1. Proteins and peptides

DnaK was purified as described previously [15]. The stock solution was stored at −80°C in 50 mM Tris–HCl, 10 mM 2-mercaptoethanol, 1 mM EDTA, 10% (v/v) glycerol, pH 8.0. The nucleotide content of the preparation was less than 0.1 mol/mol DnaK [15]. For experimentation, samples of DnaK were transferred into assay buffer (25 mM HEPES/KOH, 100 mM KCl, 10 mM $MgCl_2$, pH 7.0) with NAP-5 columns (Amersham Pharmacia Biotech). The protein concentration was determined photometrically with a molar absorption coefficient $\varepsilon_{280} = 14\,500\ M^{-1}\ cm^{-1}$ [16]. DnaJ, prepared as reported previously [17], was a gift from Dr. H.-J. Schönfeld, Basel, Switzerland. The stock solutions were stored at −80°C in 50 mM Tris–HCl, 100 mM NaCl, pH 7.7. RepA protein was purified as described [18].

Peptides p*kj* (*N*-acetyl-ALLLSAPRRSGGSGSGGSGGSGSGSG-SGSGSrraaslllac) and p*jk* (*N*-acetyl-rraaslllacSGGSGSGGSGGSGSG-GSGSGSGALLLSAPRR) were custom-synthesized with a purity of >90% by Anawa, Switzerland. Peptides ala-p5 (ALLLSAPRR) and D-p5 (*N*-acetyl-rraaslllac) were synthesized with a purity of >90% by Mimotopes and Chiron, respectively. The concentration of their stock solutions was 1 mM in 20 mM dithiothreitol. Peptides were labeled with the environmentally sensitive fluorophore acrylodan (6-acryloyl-2-dimethylaminonaphthalene, from Molecular Probes) at the sulfhydryl group or at the α-amino group (in the case of peptide ala-p5), and purified as described previously [19]. The labeled peptides were stored as stock solutions in 10% (v/v) acetonitrile.

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Abbreviations: Hsp70, 70-kDa heat shock protein; Hsp40, 40-kDa heat shock protein; acrylodan, 6-acryloyl-2-dimethylaminonaphthalene; a-, acrylodan-labeled

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2.2. Determination of kinetic constants

The binding rate constants of DnaJ for acrylodan-labeled peptides were determined with a Spex Fluorolog spectrofluorimeter in a 1 cm×1 cm cuvette [13]. The acrylodan-labeled chimeric peptides were first incubated with 2 μM DnaK in assay buffer without ATP to reach the binding equilibrium; subsequently, DnaJ was added at different concentrations. For fast kinetic measurements with DnaK in the presence of ATP, an SX18 MV stopped-flow device from Applied Photophysics served to record the changes in intrinsic fluorescence of DnaK (excitation at 290 nm, bandpass 15 nm; emission high-pass filter 320 nm). All experiments were performed in assay buffer at 25°C and were started by mixing equal volumes (70 μl each) of the two reaction solutions. All traces were obtained by averaging at least six measurements. The concentrations indicated are final concentrations after mixing, if not indicated otherwise. The reaction traces were fitted with single-exponential functions and evaluated with the software provided by the manufacturer of the instrument.

2.3. ATPase assays

The ATPase activity of DnaK was measured under single-turnover conditions as described [12]. The ATP and ADP stock solutions (100 mM disodium salt, pH 7.0) were stored in small portions at −80°C. [2,5′,8-^{3}H]Adenosine 5′-triphosphate ammonium salt (41.0 Ci/mmol) was purchased from Amersham Pharmacia Biotech. All experiments were performed in assay buffer at 25°C. The ADP product was separated from ATP by thin-layer chromatography and the radioactivity of ADP and ATP was determined with a liquid-scintillation counter. The data were analyzed with Sigmaplot 5.0 (from SPSS).

3. Results and discussion

3.1. Binding of peptides to DnaK and DnaJ

Previous studies have shown that peptides composed of D-amino acids bind exclusively to DnaJ [13,14]. In this study, both peptides p*jk* and p*kj* were designed to contain the same DnaK/DnaJ-binding motif *k* (ALLLSAPRR) and the same DnaJ-binding motif *j* composed of D-amino acid residues (rraaslllac). Motif *k* is derived from the prepiece of chicken mitochondrial aspartate aminotransferase [20]; it is bound by DnaK (K_d = 63 nM and K_d = 4.2 μM in the absence and presence of ATP, respectively [19,21]) as well as by DnaJ (K_d = 0.36 μM independent of ATP [10]). Because both DnaJ and DnaK prefer ligands containing mainly apolar residues [14], it is hardly possible to design a DnaK-binding motif that does not bind DnaJ. In the chimeric peptides p*jk* and p*kj*, the positions of the two chaperone-binding motifs are mutually exchanged and are connected by a long serine/glycine linker. Peptides ala-p5 and D-p5, which possess the same sequences as the DnaK-binding motif *k* and the DnaJ-binding motif *j*, respectively, served as controls.

Table 1
Binding and release rate constants and dissociation equilibrium constants of DnaK for peptides in the presence and absence of ATP

Peptide	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_d (μM)
In the presence of ATP:			
ala-p5 (*k* motif)	1 050 000	3.9	3.7
p*jk*	1 050 000	2.7	2.6
p*kj*	1 030 000	3.0	2.9
In the absence of ATP:			
a-p*jk*	7 300	0.003	0.4
a-p*kj*	6 800	0.0025	0.4

The values of k_{on} and k_{off} in the presence of ATP were calculated from the data shown in Fig. 1B. The given values are the means from three independent experiments. K_d values were calculated from the k_{off} and k_{on} values. The values in the absence of ATP were determined with fluorescence-labeled peptides under the same conditions except that a conventional spectrofluorimeter instead of a stopped-flow instrument was used.

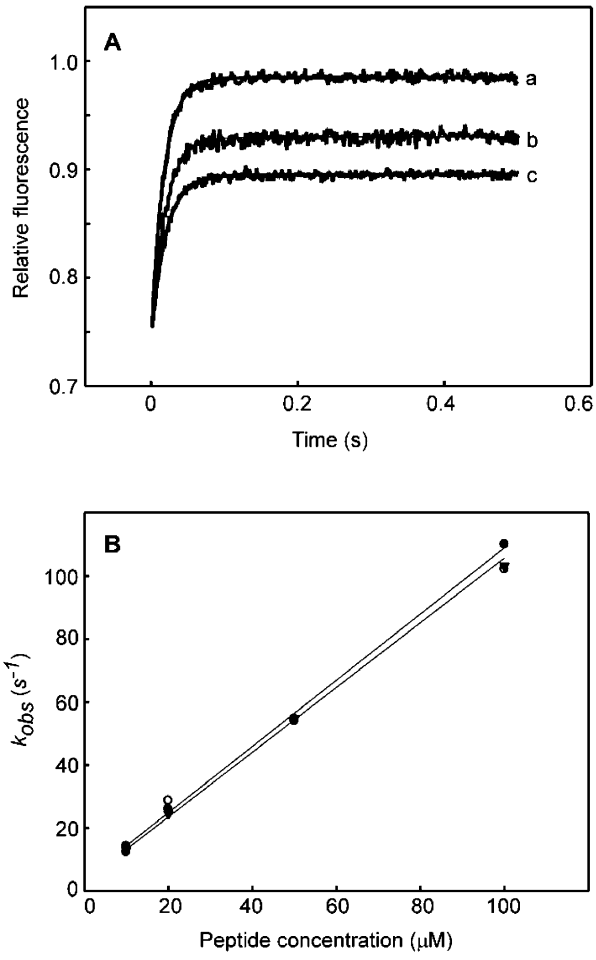

Fig. 1. Kinetics of peptide binding to ATP·DnaK. A: The rates of complex formation between peptides and ATP·DnaK were determined by monitoring the increase in intrinsic fluorescence with a stopped-flow device (for details, see Section 2). The reaction was started by mixing [1 μM DnaK+5 mM ATP] with [20 μM peptide+5 mM ATP]. The concentrations indicated are those after mixing. The solution in the first syringe was equilibrated for at least 2 min before the experiments. The pseudo-first-order rate constants k_{obs} with peptides p*jk*, p*kj* and ala-p5 were 22 (±2) s^{-1}, 24 (±2) s^{-1} and 27 (±2) s^{-1}, respectively. Trace a, p*jk*; trace b, p*kj*; trace c, ala-p5. B: The values of k_{obs} of complex formation of ATP·DnaK with p*jk* (○), p*kj* (▼) and ala-p5 (●) were plotted as a function of peptide concentration. The rates were measured in experiments as shown in A. The values of k_{on}, k_{off} and K_d calculated from these data are given in Table 1.

Complex formation of peptides with ATP-liganded DnaK was followed by the change in tryptophan fluorescence of DnaK. DnaK contains a single tryptophan residue in its ATPase domain; binding of a peptide to ATP-liganded DnaK increases tryptophan fluorescence independently of ATP hydrolysis [21,22]. The kinetic traces followed single-exponential functions (Fig. 1A). The plots of the k_{obs} values versus the concentrations of peptides p*jk* and p*kj* were linear (Fig. 1B). The on (k_{on}) and off rate constants (k_{off}) were derived from the slope and the intercept of the plots, respectively. The calculated K_d values for the two chimeric peptides and peptide ala-p5 were very similar to one another (Table 1), indicating that the *j* motif and the linker do not affect the binding of motif *k* to DnaK.

Complex formation of the chimeric peptides with DnaJ was

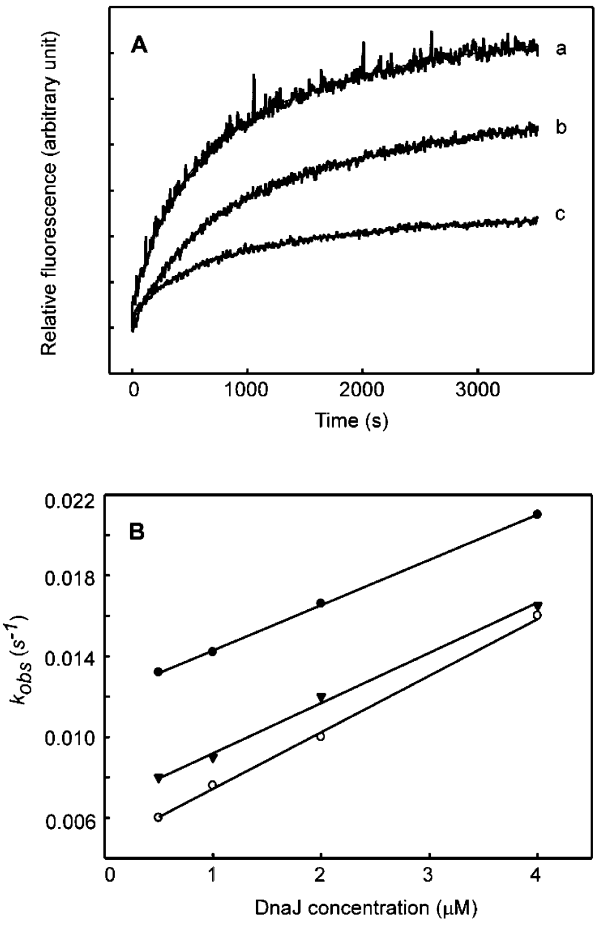

Fig. 2. Kinetics of peptide binding to DnaJ. A: The binding of the acrylodan-labeled peptides a-p*jk*, a-p*kj* and a-D-p5 to DnaJ was monitored by the increase in acrylodan fluorescence. Before the addition of DnaJ (1 μM), the acrylodan-labeled peptide (50 nM) was incubated with DnaK (2 μM) for 90 min to reach equilibrium. The traces were fitted to a double-exponential function. Trace a, a-p*jk* with $k_{obs} = 0.008$ s^{-1}; trace b, a-p*kj* with $k_{obs} = 0.009$ s^{-1}; trace c, a-D-p5 with $k_{obs} = 0.014$ s^{-1}. B: The values of k_{obs} of the first phase of complex formation of DnaJ with a-p*jk* (○), a-p*kj* (▼) and a-D-p5 (●) are plotted as a function of the concentration of DnaJ. The rates were measured in experiments as shown in A. Under the conditions of the experiments shown in A, the amplitude of the first phase was 75% of the total for p*jk*, 65% for p*kj*, and 59% for a-D-p5.

monitored by the fluorescence change of the acrylodan label upon binding to the co-chaperone [13]. Because DnaJ binds both motifs *k* and *j*, the chimeric peptides were pre-incubated with DnaK to suppress the binding of DnaJ to motif *k*. The kinetic traces with both chimeric peptides followed double-exponential functions, similar to the trace with peptide a-D-p5 in the absence of DnaK in the control experiment (Fig. 2A). The amplitudes of the first phase were considerably larger than those of the second phase and their rates proved to be a linear function of the concentration of DnaJ (Fig. 2B). The on (k_{on}) and off rate constants (k_{off}) were derived from this plot (Table 2). The association rate constants of DnaJ for both chimeric peptides and peptide a-D-p5 were very similar. Apparently, neither DnaK that is bound to the *k* motif of the chimeric peptides nor the linker interferes with the binding of the *j* motif to DnaJ. However, the k_{off} values of the chimeric peptides were only half of that of peptide a-D-p5. The retarded release is probably due to the interaction of DnaJ with DnaK [23,24] that is bound to the *k* motif of one and the same chimeric peptide.

Table 2
Binding and release rate constants and dissociation equilibrium constants of DnaJ for peptides

Peptide	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_d (μM)
a-D-p5 (*j* motif)	2200	0.012	5.5
a-ala-p5 (*k* motif)[a]	11800	0.0042	0.36
a-p*jk*	2540	0.0047	1.9
a-p*kj*	2400	0.0067	2.8

The values of k_{on} and k_{off} were calculated from Fig. 2B. The rate of complex formation was determined with a spectrofluorimeter at a constant concentration of the acrylodan-labeled peptides (50 nM) and a varying concentration of DnaJ from 0.5 to 4 μM. Before the addition of DnaJ, the peptides were incubated with 2 μM DnaK for 90 min to reach equilibrium. 2 μM DnaK is at saturating concentration for both a-p*jk* and a-p*kj* in the absence of nucleotide (K_d 40 nM and 38 nM, respectively; data not shown). The K_d values were calculated from the k_{off} and k_{on} values.
[a]Data are taken from [10].

3.2. Stimulation of ATPase activity of DnaK by DnaJ in the presence of the chimeric peptides

We have recently proposed a mechanism for the targeting action of DnaJ. ATP·DnaK and DnaJ bind to different segments of one and the same polypeptide chain forming $(ATP{\cdot}DnaK)_m{\cdot}substrate{\cdot}(DnaJ)_n$ complexes; in these ternary complexes efficient *cis*-interaction of the J-domain of DnaJ with DnaK is favored by their propinquity and triggers the hydrolysis of DnaK-bound ATP, converting DnaK to its ADP-liganded high-affinity state and thus locking it onto the substrate polypeptide [10]. The recent observation that D-peptides inhibit the refolding of denatured luciferase by the DnaK/DnaJ/GrpE chaperone system ($EC_{50} \approx 1.0$ μM for D-peptide RI 1–10 [11]) has emphasized the functional signifi-

Table 3
Effects of ligands and DnaJ on the rate of hydrolysis of DnaK-bound ATP

Additions	Concentration of substrate (μM)	k_{cat}' (s^{-1})	Relative rate
None	–	0.00025	1
DnaJ	–	0.006	24
ala-p5	50	0.006	24
D-p5	50	0.0003	1
ala-p5+D-p5	50/50	0.006	24
ala-p5+DnaJ	50	0.025	100
D-p5+DnaJ	50	0.005	20
ala-p5+D-p5+DnaJ	50/50	0.028	110
p*jk*	50	0.005	20
p*kj*	50	0.005	20
p*jk*+DnaJ	10	0.078	310
p*kj*+DnaJ	10	0.084	340
p*jk*+ala-p5+DnaJ	10/50	0.024	96
p*kj*+ala-p5+DnaJ	10/50	0.024	96
p*jk*+D-p5+DnaJ	10/100	0.028	110
p*kj*+D-p5+DnaJ	10/100	0.028	110
RepA	50	0.007	28
RepA+DnaJ	5	0.134	540
RepA+DnaJ	10	0.20	800
RepA+ala-p5+DnaJ	5/50	0.022	88
RepA+D-p5+DnaJ	5/100	0.037	150

ATPase activity was determined under single-turnover conditions at 25°C as described [12]. The final concentrations of DnaK and DnaJ were 1 and 0.1 μM, respectively (for details, see Section 2).

cance of this targeting mechanism. Because D-peptides compete with L-peptides for the same binding site in DnaJ but do not bind to DnaK, the inhibition of the chaperone action is attributed to the binding of the D-peptide to DnaJ which is thus precluded from forming (ATP·DnaK)$_m$·substrate·(DnaJ)$_n$ complexes. Apparently, simultaneous binding of DnaJ and DnaK to one and the same target polypeptide is essential for effective chaperone action.

In the absence of DnaJ, both p*jk* and p*kj* stimulated the single-turnover ATPase activity of DnaK 20-fold in comparison with the intrinsic ATPase activity, i.e. to the same degree as the control peptide ala-p5 (motif *k*) (Table 3). However, in the presence of DnaJ, the chimeric peptides stimulated the ATPase activity of DnaK more than 300-fold while ala-p5 increased the activity only 100-fold. The chimeric peptides thus exert a similar effect as the protein substrate RepA, which has been reported to form a ternary complex with both chaperones [25] and stimulated the ATPase activity of DnaK in the presence of DnaJ up to 800-fold (Table 3). In the control experiment, peptides ala-p5 (*k* motif) plus D-p5 (*j* motif) stimulated the ATPase activity of DnaK only to the same level as peptide ala-p5 alone in the presence of DnaJ. Apparently, the enhanced stimulatory effect of the chimeric peptides depends on the formation of ternary (ATP·DnaK)·(p*jk*/p*kj*)·DnaJ complexes. Upon addition of either peptide ala-p5 or D-p5 at 10-fold higher concentration than that of the chimeric peptide, the stimulatory effect of both chimeric peptides and RepA in the presence of DnaJ decreased to the same level as that of peptide ala-p5. Apparently, the short, single-motif peptides ala-p5 and D-p5 obstructed the formation of (ATP·DnaK)·(p*jk*/p*kj*)·DnaJ and (ATP·DnaK)·RepA·DnaJ complexes by binding to the substrate-binding sites of DnaK and DnaJ. All results on peptide binding and its competitive inhibition (Table 3) are consistent with the notion that both p*jk* and p*kj* form ternary complexes allowing the *cis*-action of DnaJ on DnaK. The interactions of the chimeric peptides with DnaK and DnaJ thus resemble the interactions of unfolded protein substrates with the chaperones [10].

DnaK binds hydrophobic peptides in extended conformation [2,26]. DnaK-binding motifs in protein sequences occur statistically every 36 residues and the peptide-binding site of DnaK accommodates seven amino acid residues [2,27]. The binding motif for DnaJ consists of sequences of eight amino acid residues [14]. The serine/glycine linker between the *k* and *j* motifs in the chimeric peptides is 22 residues long, which corresponds to ~70 Å and when compared with the above data, seems long enough to allow simultaneous binding of DnaK and DnaJ. Both chimeric peptides (p*jk* and p*kj*), which possess the same binding motifs for DnaK and DnaJ in different order, formed ternary complexes with DnaK and DnaJ. The *cis*-action of DnaJ was equally effective in the presence of either chimeric peptide. The somewhat diminished *cis*-effect observed with the chimeric peptides in comparison with protein substrates such as RepA (Table 3), denatured rhodanese [10], σ^{32} and denatured luciferase [9,11] can thus hardly be attributed to steric hindrance, e.g. resulting from a too short linker. The reason for the difference in quantity between the effect of the chimeric peptides and that of a protein substrate is unclear as yet. Possibly, the multiple binding sites for DnaJ and DnaK in a polypeptide substrate result in a kinetically more favorable situation for interaction between DnaK and DnaJ.

One of several known reactions that the DnaK chaperone system carries out in non-stress situations is the maintenance of plasmid mini-P1 [28,29]. In vitro, DnaK, DnaJ, and GrpE are required for DNA replication of mini-P1; the sole function of the chaperones is to convert inactive RepA dimers into active monomers [25,30,31] and thus to trigger its binding to the origin of replication on DNA. DnaK and DnaJ act on dimeric RepA by recognizing and binding to separate segments of RepA [32]. The present results indicate that the formation of ternary (ATP·DnaK)·RepA·DnaJ complexes facilitates the interaction of the J-domain of DnaJ with the ATPase domain of DnaK. RepA is thus another example besides σ^{32} [9] of a native protein with which the *cis*-effect of DnaJ on DnaK can be observed as it has been found with unfolded proteins such as denatured luciferase [9], rhodanese [10] and now with the chimeric peptides.

3.3. Concluding remarks

The two most important findings of this study are: (1) the DnaJ-stimulated ATPase activity of DnaK in the presence of the chimeric peptides is three times higher than in the simultaneous presence of the two corresponding separate peptides with the same DnaK- and DnaJ-binding motifs (ala-p5 and D-p5, respectively); (2) the *cis*-interaction of DnaJ and DnaK in the presence of chimeric peptides is inhibited both by ala-p5 and by D-p5. From findings (1) and (2), we conclude that the simultaneous binding of DnaK and DnaJ to one and the same chimeric peptide results in a kinetically favored *cis*-effect of DnaJ on DnaK. Chimeric peptides possessing both a DnaK- and DnaJ-binding site may thus serve as minisubstrates that behave, at least with respect to the interaction between DnaJ and DnaK, quite similarly to unfolded proteins and special native proteins such as RepA and σ^{32}. RepA and σ^{32} [25,33] are proteins designed to interact in their native conformation with the DnaK/DnaJ/GrpE system. Formation of ternary (ATP·DnaK)·substrate·DnaJ complexes that results in *cis*-interaction of DnaJ and DnaK [10,11] seems to be an integral feature of the interaction of Hsp70 chaperones with both unfolded proteins and native-state proteins designed to interact with the chaperone system.

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